Teaching Tools in Plant Biology Lecture Notes

Rhythms of Life: The Plant Circadian Clock

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Abstract

This teaching tool explores circadian rhythms in plants. This is an exciting and fast-moving area of research, which requires us to think in terms of temporal biological dynamics, interconnected networks of cellular components, and the relationship between plant molecular biology and environmental adaptation. We present this topic as a series of concepts illustrated by examples, including the architecture of circadian clocks, the connections between the oscillator and circadian-regulated processes such as metabolism and control of flowering time, and consider how understanding circadian rhythms could lead to crop improvement. We also explain some of the techniques used to investigate circadian biology, as many of these may be unfamiliar. We do not describe each component of the circadian oscillator; there are so many genes and mechanisms involved that this resource would become unintelligible and not be useful to an undergraduate audience. Instead, we consider it more important to think in terms of the overall organisation and principles, rather than becoming lost in the details of individual components. For those interested in finding out more, there are many excellent reviews on circadian rhythms, some of which we highlight at the end of the article.

Article contents

Introduction

The rotation of the Earth on its axis causes cycles of day and night. This causes repetitive daily changes in the environment that present living organisms with a variety of challenges. During each 24 h day, there are large fluctuations in light intensity and temperature, which also lead to daily fluctuations in other aspects of the environment such as humidity and predator behaviour. The requirement to co-ordinate biological processes with environmental light-dark and temperature cycles and biological processes, has led to the evolution of circadian clocks (from the Latin *circa* meaning about, and *dies* meaning day). Circadian rhythms are defined as biological oscillations having a cycle of about 24 hours, which persist in the absence of external signals. The persistence of the rhythms in conditions of continuous light (or dark) and temperature indicate that they are driven by an endogenous biological process. For example, rodents have higher levels of wheel running activity at night under normal light-dark cycles. However, if kept in continuous darkness there are still rhythms of activity, but the period of these oscillations is regulated by the internal biological oscillator so may not be exactly 24 hours. Human physiology is controlled extensively by an internal circadian oscillator. The impact of the circadian oscillator is felt most keenly in jet-lag, where the discrepancy between the time of day predicted by the internal oscillator and the external conditions results in physiological stress. Circadian oscillators (circadian clocks) have evolved multiple times independently, and are found in organisms from all kingdoms of life.

Plant circadian biology has a long history; for example, it the ancient Greeks are thought to have been aware of rhythmic processes in plants. Detailed measurements of plant circadian rhythms were made in 1729 by the French biologist Jean-Jacques d'Ortous de Marian in the heliotropic plant *Mimosa pudica*. He observed changes in the folding of the leaflets of *Mimosa* under constant environmental conditions. These leaf movements were referred to as 'sleep' movements, and occur in a range of different plants. Charles and Francis Darwin also recorded daily changes in leaf position in 'The Power of Movement in Plants' (1880). In 1751 Carl Linneaus proposed a model for a 'Floral Clock', whereby the predictable opening and closing of flowers at particular times of day could be used to indicate the time. Uncovering the molecular bases for these rhythms has been the focus of a huge amount of research in recent years, and we now have a good understanding of how the circadian clock regulates many aspects of plant physiology, molecular biology and development.

The architecture of circadian clocks

Most of the research on the functioning of plant circadian clocks has been performed in the model plant *Arabidopsis thaliana.* Circadian clocks in plants, animals, insects, and fungi share similar properties and features, although the specific genes involved are different. Most circadian oscillators are based on transcriptional regulation, and the importance of post-transcriptional regulation is becoming increasingly understood. The circadian clock in cyanobacteria is functionally different to eukaryotic oscillators because it is based mainly upon post-translational regulation. Circadian clocks may be an interesting example of convergent evolution, because they are thought to have evolved independently on a number of occasions.

The circadian network needs to have three main properties to coordinate the activities of the plant with the external environment:

1) It needs to generate a 24 h rhythm inside the cell that can be sustained in the absence of external stimuli. This is accomplished by the genes and proteins of the 'circadian oscillator' (sometimes called 'circadian clock').

2) The circadian clock needs to be kept synchronized with the environment. In other words, its phase needs to match the phase of the environment. The synchronization process is known as 'entrainment'.

3) Mechanisms must exist to link the circadian clock with aspects of the plant that have circadian rhythms. These are known as 'output pathways' because they connect the output of the clock- which is a measure of the time of day- with other aspects of plant cells. The main way the circadian clock influences the cell is by regulating daily rhythms of transcription of a large number of genes. In turn, this leads to circadian rhythms in biochemistry and physiology (see later sections of the teaching tool).

There is an additional level of complexity in that the sensitivity of both the entrainment and output pathways can change over the course of the day, which may be regulated by the circadian clock itself. This is referred to as 'circadian gating' and is discussed in detail in later sections of the teaching tool.

A useful piece of terminology that is used in circadian biology, which we use in this article, is the word 'subjective' in relation to events that occur during the day-night cycle. When a plant is placed under constant conditions, it no longer experiences dawn and dusk. However, the time of day at which dawn, dusk or night would have occurred, had the plant been in day-night conditions, is called the 'subjective dawn' 'subjective night', etc.

The circadian oscillator

NB: In this section we consider processes that give rise to circadian rhythms in plant cells. This is a rapidly moving field and checking recently published literature or reviews is recommended, as models of the circadian oscillator are updated frequently as new discoveries are made.

In general, circadian oscillators are formed from transcription-translation feedback loops. The conceptual example on the slides considers a very simple oscillator with two components, A and B. A and B are genes that encode proteins that are part of this oscillator. Here, one of the genes activates the other, and one gene represses the other, so they regulate each other in a cyclical manner. Over a day, Gene A is expressed in the morning and the protein that it produces is an activator of Gene B. Therefore, when a certain amount of Protein A has accumulated, Gene B is turned on and expressed later in the day. However, Protein B is a repressor of Gene A, so as Protein B accumulates, Gene A is switched off and Protein A levels decrease during the night. Since Protein A is required to activate Gene B, as Protein A decreases, Gene B is turned off. This releases the repression of Gene A by Protein B, and Gene A begins to increase again the following morning. The biochemical kinetics of these processes introduce rate constants and delays into the oscillator,

causing the cycle to be completed in about 24 hours. Each plant cell is thought to have its own circadian oscillator that can operate independently, but recent research has found that there is communication between the circadian oscillators of different tissue types.

Early breakthroughs in understanding the plant circadian clock focused upon interactions between the transcription factor genes *TIMING OF CAB EXPRESSION 1* (*TOC1)*, *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1)* and *LATE ELONGATED HYPOCOTYL* (*LHY)*. It was thought originally that TOC1 activates *CCA1* and *LHY* expression, and CCA1 / LHY repress *TOC1*, establishing a negative feedback loop in a similar manner to Gene A and Gene B in the hypothetical oscillator. Models of the oscillator have changed significantly since then, but at their core all contain a negative feedback loop. A significant revision to the early models is that we now know that TOC1 represses rather than activates *CCA1* / *LHY*; care should therefore be taken when reading around this subject as older models may well include inaccuracies including the structure of the *TOC1-CCA/LHY* loop.

A large number of oscillator genes have been identified through a variety of different experimental approaches. The oscillator genes are expressed at different times of day; for example *CCA1* is maximally expressed around dawn while *LUX ARRHYTHMO* (*LUX*) reaches a peak around 12 hours after dawn. Current thinking about the structure of the plant circadian clock is that it is quite complex, with several feedback loops of gene expression that interact with each other. The circadian oscillator incorporates a main feedback loop linking CCA1 / LHY with TOC1, GI and LUX / ELF3 / ELF4. There is also a morning loop involving CCA1, LHY, PRR7 and PRR9, and an evening loop involving the 'evening complex' of ELF3, ELF4 and LUX.

An underlying feature of plant circadian oscillators is that they maintain a free running period of about 24 h across a range of physiologically-relevant temperatures, meaning that the circadian period is buffered against changes in temperature. This feature is called 'temperature compensation' of the circadian oscillator. Given that the rate of enzyme activity is temperature-dependent, the robustness of the circadian oscillator to changes in temperature is an intriguing aspect of circadian regulation. Temperature compensation of the circadian oscillator appears involve temperature-dependent regulation of CCA1 and LHY expression. Several mechanisms have been identified, including roles for PRR7 and PRR9, a regulator of CCA1 transcription called FBH1, and temperature-dependent alternative splicing of RNA.

In addition to transcriptional feedback loops, there are a number of post-transcriptional control mechanisms that contribute to the correct functioning of the oscillator. These include chromatin remodelling, regulation of protein degradation by the proteasome, phosphorylation of oscillator proteins and the involvement of small signalling molecules such as cyclic ADP ribose (cADPR) and cytosolic free Ca^{2+} (see Mas 2008 for a review of post-transcriptional processes).

The complexity of the oscillator means that circadian research is moving away from identification and characterisation of individual oscillator components, and towards understanding overall emergent properties of the gene network. Mathematical modelling studies (see later sections of teaching tool) have suggested that the complexity and interconnectivity within the oscillator may provide the oscillator with greater robustness or stability in the face of many fluctuating aspects of the environment that change the regulation of components of the clock.

Entrainment pathways

Circadian clocks never have a period of precisely 24 h, so if the circadian clock was not resynchronized with the environment every day it would not predict time accurately for the plant. Additionally, entrainment allows small daily adjustments to the phase of the circadian clock to match the changes in the time of dawn that result from the progression of the seasons (see Slide 14 for an example of the seasonal changes in the time of dawn that occur in northern Europe). Several environmental signals act as entrainment cues. Red and blue light, sensed by the phytochrome and cryptochrome photoreceptors respectively, provide a strong resetting signal to the circadian clock and are important for synchronization of the circadian clock with dawn. Phytochrome A acts to regulate the clock mainly under low intensity red light, whereas Phytochrome B is important under high intensity red light. Cryptochrome 1 is important for regulation of the clock under low and high intensity blue light. It is thought that phyA and phyB act additively, whereas cry1 and cry2 act redundantly. The circadian clock component ZEITLUPE (ZTL) also senses blue light and contributes to the response of the circadian clock to light. Photosynthesis within chloroplasts produce sugars, which also entrains the circadian oscillator (see later sections of teaching tool). Temperature fluctuations can also entrain the circadian oscillator, however the molecular mechanisms of temperature entrainment are poorly understood at present.

The importance of the circadian clock in plant biology

The circadian clock influences almost all aspects of plant biology, and confers a significant selective advantage. *Arabidopsis thaliana* plants that have been mutated to abolish circadian rhythms (*CCA1*-ox) have only 53% of the biomass of wild type plants. Wild type plants also accumulate significantly less biomass when grown under either 20 or 28 hour days than under 24 hour days, indicating that the period of the endogenous oscillator must match that of the external environment for optimal growth. The correct functioning of the circadian clock therefore has very significant impacts on plant performance.

The circadian clock influences plant biology at a range of different levels. Analysis of the transcriptome indicates a significant proportion of the *Arabidopsis thaliana* genome is under circadian control. The percentage of transcripts that are expressed rhythmically varies between different studies, but meta-analyses of multiple microarray datasets indicate that around a third of the transcriptome cycles in constant conditions. Similar patterns of circadian transcript abundance have been found in other species including tomato, soybean, rice, sugarcane and poplar. A large number of genes associated with metabolism are under circadian control, indicating that the clock has a significant effect on plant biochemistry (see later sections of teaching tool). The clock also controls physiological processes such as leaf gas exchange, with stomatal being more open in the subjective day than subjective night, when plants are grown under continuous light.

Growth and development are also under the control of the circadian oscillator. Video imaging of Arabidopsis seedlings under continuous light reveals that both the elongation of the hypocotyls of seedlings, and changes in cotyledon position, are rhythmic. Whether the clock directly controls cell division in higher plants is an open question, but circadian control of water and carbon availability contributes to rhythmic patterns of growth. Gibberellin and auxin-dependent growth is also regulated by the oscillator through a variety of mechanisms. Developmental transitions such as the

initiation of flowering are also under the control of the circadian clock; many circadian clock mutants flower either earlier or later than wild type plants when grown under long days (see later section of teaching tool for details of mechanisms). It is clear that the circadian clock controls many different aspects of plant biology, and is essential for optimum plant performance.

The circadian clock provides organisms with a fitness advantage, explaining why circadian oscillators have evolved independently multiple times across different kingdoms of life. In plants, this advantage can be demonstrated through competition experiments in the model plant *Arabidopsis thaliana*. When plants are grown under 20 hour long days (10 hours light, 10 hours dark), mutant plants with a short circadian period (*toc1*-2) accumulate more biomass than mutants with a long circadian period (*ztl*-27; see later sections of teaching tool for definition circadian period). However, if grown under 28 hour long days (14 hours light, 14 hours dark) the situation is reversed, so mutant plants with a long circadian period outcompete those with a short period. The same pattern was seen when mortality rates were compared, indicating that having an internal circadian oscillator that matches the dynamics of the external environmental conditions confers a significant fitness advantage.

Investigating circadian rhythms in the laboratory

Properties of circadian rhythms

Circadian rhythms are self sustaining in the absence of signals from the environment. Therefore, a common way to study circadian rhythms is to place the plant under constant conditions- constant temperature and constant light or darkness- and monitor the circadian regulated aspect of physiology or biochemistry that is of interest. The plant is typically grown for a period time under cycles of day and night, then transferred to constant conditions. Under constant conditions, the circadian clock is said to 'free run' and the experimental conditions are sometimes called 'free running conditions'. For example, to study circadian rhythms of photosynthesis, the plant might be cultivated in 24 h light-dark cycles for several weeks before being moved to continuous light for the measurement of photosynthetic CO_2 uptake.

Circadian rhythms have a number of properties that can be quantified and are often used during studies of plant circadian biology. In continuous conditions, the circadian period is the time taken by the rhythm to complete one full oscillation. This is typically about 24 h in wild type plants. The phase is the time after dawn when a specific point in the cycle occurs, such as the peak. For example, the rhythm could have a dawn phase or a dusk phase, depending on the property that is being investigated. The amplitude of the rhythm is the difference between the average (center) point in the oscillation and the maximum or minimum of the oscillation. These properties can be quantified with a variety of analytical tools, such as Fast-Fourier Transform.

The reason it is useful to measure these properties of circadian rhythms is because they can change depending on the state of the circadian clock. For example, mutating genes that encode parts of the circadian oscillator can cause circadian rhythms to adopt a period that is longer or shorter than 24 h. Similarly, mutations to other parts of the circadian clock can change its phase or its amplitude. Measuring these properties has allowed important progress to be made in the identification of

components of the circadian clock, understanding how the components interact, and understanding which aspects of plant physiology are controlled by which component of the circadian clock. For example, the central oscillator component TOC1 was first identified in a forward genetic screen for plants with altered circadian periods of *CAB2:LUCIFERASE* activity (see below).

Common measurement techniques for studying circadian rhythms

To gain molecular insight into circadian rhythms it is common to collect a time-course of samples of plant tissue from which mRNA is isolated to monitor circadian changes in mRNA transcripts encoding proteins. Depending on the transcript studied, this can be used to investigate- for example- the functioning of the circadian clock, the control of metabolism by the clock, or provide a read-out of the operation of a signalling pathway associated with the clock. Experiments often use quantitative RT-PCR to measure the amount of transcript, but also studies have monitored circadian changes in either all or a substantial proportion of plant transcripts (the transcriptome) using microarray or sequencing methods (Harmer et al. Science 2000 is a great example of this). In a similar manner, tissue samples can be collected to monitor circadian rhythms in the quantity of certain proteins, the activity of enzymes, or the concentration of metabolites.

Circadian experiments often involve repetitive measurements at regular intervals over a long period of time. This is laborious, can involve antisocial hours- also increasing the chances of mistakes- and makes large scale experiments difficult. In addition, taking regular plant tissue samples to measure gene expression, enzyme activity or a metabolite requires considerable amounts of plant material to be grown in order to obtain enough samples through the time-course for sufficient levels of experimental replication. To address this, several non-destructive / non-invasive technologies have been developed to allow relatively straightforward monitoring of plant circadian rhythms.

One non-invasive technique involves monitoring circadian rhythms of seedling growth using a camera. The hypocotyl (stem) of seedlings grows faster at night, and an automated camera trained carefully on the young seedling can capture the rhythms in the position of the leaves that result from this pulsatile growth. Since the circadian rhythm of growth is controlled by the circadian clock, this method again provides a way to study the operation of the circadian clock. More commonly the positions of the leaves themselves can be recorded; in some species the leaves change position due to the activity of the pulvinus at the base of the leaf, whereas in others (including Arabidopsis thaliana) the 'leaf movement' response is in fact due to rhythmic patterns of growth. One advantage of this approach is that it does not require the generation of transgenic plants (see below). To see some growth rhythms of plants, check out the movies on the Plants in Motion website (http://plantsinmotion.bio.indiana.edu/plantmotion/starthere.html).

While leaf movement imaging is useful as a circadian-dependent phenotype and is commonly used to screen for or characterise circadian mutants, it gives little molecular insight into the oscillator. The non-invasive bioluminescent reporter luciferase has revolutionized plant circadian biology and underpinned the discovery of many parts of the circadian clock in the model plant *Arabidopsis thaliana*. Luciferase is an enzyme derived from fireflies that catalyzes the biochemical reaction causing fireflies to glow. If an optimized luciferase is introduced to plants by making a transgenic

strain, and the plant is supplied with the substrate of luciferase (called luciferin), the plants will emit light when the luciferase gene is expressed. The light emitted from the plant, due to the luciferase, can be detected with sensitive camera systems or a luminometer. If luciferase is expressed in plants under the control of a circadian-regulated gene promoter from the plant such as CHLOROPHYLL A/B BINDING PROTEIN 2 (*CAB2*), circadian rhythms occur in expression of luciferase (and hence there is a rhythm in the amount of light emitted from the plant). Luciferase monitoring instruments for circadian rhythms research are often automated, making experiments relatively straightforward. In a typical experiment, the plants are illuminated, then every hour or so the lights turn off, luciferase bioluminescence is measured, then the lights turn back on again, then the lights turn back on again so the plants can photosynthesize.

Advanced methods for studying circadian rhythms

Circadian bioluminescence imaging has been adapted to address specific questions in plant circadian biology. This type of imaging typically monitors the circadian rhythms in a whole seedling by measuring luciferase bioluminescence from entire plants. However, by using a particularly sensitive camera and appropriate lens, the variation in circadian rhythms across single leaves can be measured. Circadian rhythms can even be measured in single tissue types by using a 'split luciferase' or 'bimolecular luminescence complementation' technique. In this, one half of the protein is expressed with a tissue specific promoter (e.g. vascular tissue only) and the other half a circadian regulated promoter- and the two halves of the enzyme only come together to emit light in the chosen tissue at the right time! It is even possible to measure circadian rhythms in single cells of a leaf by firing microscopic beads coated with luciferase gene at the leaf- they only penetrate some cells, which are the ones that subsequently glow and from which the rhythm can be measured.

From the slides about the structure and function of the molecular circadian clock, you will see that it is extremely complex. There are multiple feedback loops, many interconnected components with both negative and positive regulation steps, and both transcriptional and post-transcriptional regulation processes. Considering the number of components, it is difficult- if not impossible- to envisage with a diagram how the plant circadian clock functions. As a result, circadian biologists have turned to mathematical modelling to deepen their understanding of the plant circadian clock. Mathematical modelling has helped to identify gaps in our knowledge of the circadian clock, test the accuracy of our theories concerning the operation of the circadian clock, and provide adaptive explanations for certain properties of the clock, such as its complexity. In molecular plant biology, the field of circadian rhythms was one of the first to use mathematical modelling to understand a complex gene network and it could be argued that circadian rhythms researchers had a pioneering role in the more widespread adoption of 'systems biology' approaches in plant sciences.

The circadian clock and plant metabolism

Primary metabolism

Plant metabolism undergoes dramatic shifts under each day-night cycle, with photosynthesis dominating during the day and starch degradation and nutrient assimilation occurring at night. This

is not simply a response to changes in light availability, but is also under the control of the circadian clock.

The first circadian transcriptome studies revealed extensive control of metabolism by the circadian oscillator. It regulates the transcription of large number of enzymes of primary metabolism, including chlorophyll biosynthesis, photosynthetic electron transport, starch synthesis and degradation, nitrogen and sulphur assimilation (see Farré and Weise, 2012 for review). In many cases the peak in RNA abundance precedes that of the actual physiological process that the enzyme is involved in; for example the expression of chlorophyll biosynthesis genes occurs at the end of the night, anticipating the onset of dawn. In contrast, the peak of genes associated with starch catabolism is around subjective dusk.

Care should be taken not to over-interpret circadian transcriptome analyses as there are several examples where individual transcripts have a circadian rhythm but there is no corresponding circadian rhythm in protein level or catalytic activity. This does not necessarily mean that circadian rhythms of transcription are without physiological significance; it may be that circadian rhythms in gene expression compensate for patterns in protein degradation, so the role of the oscillator is to maintain a constant level of protein. Alternatively there may be additional levels of post-transcriptional regulation that contribute to control of metabolism.

Metabolite concentrations in circadian mutants differ from those in wild type plants, indicating a link between the circadian oscillator and metabolism suggested by the transcriptome data. A metabolite analysis of a *prr9/7/5* triple mutant found that the concentration of citric acid cycle (TCA cycle/Kreb's cycle) intermediates (e.g. malate, fumarate) was significantly higher in the mutant than in wild type plants. This can be partially explained by the reduced levels of fumarase and 2-oxoglutarate dehydrogenase expression in these mutants. The triple mutants also have high levels of shikimate, which is a precursor molecule for many secondary metabolites. The metabolic phenotype of the *prr* triple mutant was distinct from that of CCA1-ox despite both mutants being arrhythmic, indicating different parts of the oscillator have different regulatory functions in metabolism.

One of the roles of the oscillator is to allow plants to predict light availability. At night plants are unable to photosynthesise, so are in danger of starvation. During the day starch is synthesised as a temporary store of carbon, which is then degraded through the night to support respiration and growth. The rate of starch degradation is tightly controlled so that plants exhaust 95% of their starch by the end of the night. The plant can adjust this rate to reflect the length of the night, so that starch degradation occurs more slowly in long nights. Remarkably, the plant can also adjust the rate of degradation immediately in response to an unexpected early onset of night. When plants grown under 12h light: 12 h dark cycles experience darkness only 8 hours after dawn they reduce the rate of nocturnal starch metabolism, thereby preventing starvation at the end of the night. *cca1/lhy* mutants degrade starch 35% faster than wild type plants, meaning they run out of starch 3-4 hours before the end of the night and enter a period of starvation. The inability to regulate starch metabolism therefore also contributes to the reduced growth of circadian clock mutants.

As metabolism in plants is split between different organelles it is also important to consider how the oscillator is spatially organised within the cell. There are circadian rhythms of gene expression within

the chloroplast, which seem to controlled by the nucleus. Gene expression of the nuclear-encoded sigma factor SIG5 is controlled by the circadian oscillator, with maximal expression around dawn. SIG5 is then imported into the chloroplast where it forms part of a chloroplast RNA polymerase to control the expression of plastid genes including *psbD*, which encodes the D2 protein of Photosystem II.

Early genetic models of the plant circadian system proposed that light and temperature entrained the oscillator, which then controlled outputs such as metabolism and stomatal movements. However, it is now clear that metabolic processes also regulate the circadian oscillator, so the connection between the clock and metabolism is infact bi-directional. Young seedlings grown in the absence of sucrose have very low amplitude circadian oscillations of *CCA1:luc* and *CAB2:luc* in continuous dark, but these rhythms increase in amplitude in the presence of exogenous sucrose. The restoration of rhythmicity is dependent on the central oscillator component GIGANTEA. Application of metabolically active sugars (sucrose, glucose, fructose) shortens the period of circadian rhythms of plants grown in continuous low light. Externally applied sugar entrains the central oscillator, but rhythms entrained to sugar have different characteristics to those entrained by light, indicating that sugar is an independent entrainment signal in the circadian network. Redox status and the co-enzyme NAD^+ also contribute to plant circadian rhythms. The relationship between the clock and primary metabolism is therefore more complex than the early models suggested, with metabolism both being controlled by and controlling the central oscillator.

Secondary metabolism

Secondary metabolism is also under the control of the oscillator. Secondary metabolites are molecules that are not required directly for the survival of the organism. Secondary metabolites play a variety of roles in plants, including pigments and herbivore defence compounds. Floral scent molecules are volatile secondary metabolites, having a key role in attracting pollinators. For example, *Petunia hybrida* cv. Mitchell releases volatile compounds at night to attract pollinators such as hawkmoths, and this has been shown to be under the control of the circadian oscillator. There is a circadian rhythm in the production of volatiles such as methyl benzoate in continuous darkness, but no such rhythm occurs in continuous light. Volatiles are synthesised from phenylalanine via the shikimate, phenyl-propanoid and benzenoid pathways. Many of the enzymes and transcriptional regulators in this pathway are under circadian control at the level of transcription, with maximal expression occurring during the subjective night. If the clock gene LHY is constitutively overexpressed in *Petunia hybrida*, rhythms of volatile emission are disrupted.

The circadian clock also plays a role in the interactions between plants and herbivores. In one study looking at the interaction between Arabidopsis and cabbage loopers it was found that if the plants and insects were entrained to the same light-dark cycles, the plants were able to resist herbivory under continuous darkness. However, if the plants and insects were entrained to opposite conditions, the plants were vulnerable to attack by the caterpillars. The insects have a strong circadian rhythm of feeding, with maximal feeding occurring during the subjective day. Plant herbivore defences are induced by jasmonates, which were found to accumulate during the subjective day. The plant is therefore actively protecting itself during the time of maximal insect feeding, representing an additional benefit to having a circadian clock. Through influencing plant-animal interactions it is

therefore likely that the circadian clock contributes towards ecological dynamics, although this has not yet been systematically been investigated.

The circadian clock provides timing information to control photoperiodic flowering

Annual changes in photoperiod provide an environmental cue that is used by plants to detect changes in the seasons. Many plants use the changing photoperiod to control the season of flowering. For example wheat (*Triticum aestivum*) flowers in late spring when the days become longer, whereas rice (*Oryza sativa*) flowers in late summer when days become shorter. Photoperiod sensitive plants can be divided into long day and short day plants. Long day plants flower when the dark period is shorter than a particular duration, and will also flower if a long night is interrupted by a short exposure to light. Short day plants flower when the dark period is longer than the critical length, and are unaffected by interruptions to the night. Some plants are obligately photoperiodic (they only flower under a specific photoperiod), while others are facultatively photoperiodic (flowering is stimulated by photoperiod but the plant will eventually flower even under non-flowering photoperiods). Here, we consider specifically the role of circadian regulation in the seasonal regulation of flowering, though it is important to remember that other environmental cues (e.g. temperature, abiotic stress) also influence the transition to flowering.

The "external coincidence model" was first proposed by Erwin Bünning in 1936 as way to explain photoperiod-dependent flowering in long days. In the simplest version of the model there are two components; a circadian dependent regulator which has its peak of expression between 10-14 hours after dawn, and a downstream floral inducer. Flowering is only induced when expression of the circadian dependent regulator and light coincide. In short days the expression of the regulator is low during the day so the plant continues in vegetative growth. In long days the regulator is expressed highly in the afternoon, therefore light and the regulator coincide and the floral inducer is activated.

The molecular basis of this process is now well characterised, with at least a dozen different proteins playing a role in the mechanism. Flowering is a tightly controlled event which centres on the regulatory protein FLOWERING LOCUS T (FT). FT is regulated at the level of transcription by a number of different pathways including the photoperiodic pathway, and when its expression is stimulated the protein moves from the leaves to the shoot apical meristem to induce flowering. One activator of FT is CONSTANS (CO), a zinc-finger transcriptional activator which is indirectly regulated by the circadian oscillator. These two proteins can be integrated into the external coincidence model, where CO represents the circadian dependent regulator and FT the floral inducer.

CONSTANS expression is rhythmic, with the maximal level of *CO* mRNA being ~12-14 hours after dawn. However, CO protein is unstable in the dark as it is targeted for degradation by the E3 ubiquitin ligase CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1). This provides a mechanism through which photoperiod can be detected:

- In short days the peak in *CO* mRNA levels occurs at night, and the protein does not accumulate, hence FT is not induced and the plant stays in the vegetative state.
- In long days the peak of *CO* mRNA occurs during the light, and CO protein is able to accumulate. The stabilisation of CO protein in the late afternoon allows the expression of *FT*, which can then travel to the meristem and induce flowering.

The same basic coincidence model is conserved between Arabidopsis and crop species, including rice which is a short day plant. In rice the equivalent of *CO* is *Heading-date 1* (*Hd1*), and the equivalent of *FT* is *Heading-date 3a* (*Hd3a*). The major difference between the two models is that while in Arabidopsis CO *activates* FT expression, in rice Hd1 is a *repressor* of Hd3a. This means that when coincidence between Hd1 expression and light occurs in long days the expression of Hd3a is suppressed, and the plant remains in the vegetative state.

Circadian gating

An important principle in circadian biology is circadian gating. Circadian gating is the process whereby the circadian clock adjusts the sensitivity or response of another signalling pathway, so that the outcome from the pathway depends on the time of day. In practice this means that when an identical stimulus is applied at different times of day, there is a circadian rhythm of the strength of the response to the stimulus. Essentially, the clock acts as a valve on another signalling pathway.

Circadian gating acts upon the entrainment of the oscillator itself, and also the responses of plant cells to the environment. Firstly, the circadian clock gates its own response to entrainment signals. Application of an entrainment cue (e.g. light) changes the phase of the circadian clock towards dawn. Circadian gating of this response means that the clock is much more sensitive to light during the night and around dawn than during the rest of the day. This is very important, because if the circadian clock were equally sensitive to light throughout the entire day, it would be continuously reset to dawn and so unable to maintain an accurate measure of the time of day. In addition, the light intensity may change considerably over the course of the day due to cloud cover or shading by other plants, so adjusting sensitivity to light levels through the day may confer benefits to plants in the natural environment.

In addition to gating of the entrainment pathways, the circadian clock also gates the responses of other environmental responses in plants. It is thought that this ensures that the nature of the response to the environmental signal is appropriate for the time of day. Here are two examples of circadian gating:

(1) The circadian clock underlies a daily rhythm in the sensitivity of plants to cold. The CBF family of genes regulate processes that increase the freezing tolerance of Arabidopsis. When plants were exposed to 4 ^{o}C at a range of times of day, the degree of upregulation of the CBF family genes (CBF2 on the slide) has a circadian rhythm. Overall, the CBF genes are more sensitivity to upregulation by cold at 4 h and 10 h after subjective dawn, rather than during the subjective night.

(2) The circadian clock regulates a rapid response of developing seedlings to shade. During seedling establishment, rapid elongation of the hypocotyl positions the leaves optimally for photosynthesis. If the seedling is shaded by other plants, the ratio of red to far red light reduces, because vegetation absorbs red light and reflects/transmits far red. This is sensed by the seedling and causes rapid elongation of the hypocotyl, to allow the seedling to over-top its neighbours and catch the light. This

response is gated by the circadian clock, such that the hypocotyl elongates much faster when exposed to low red:far red light around subjective dusk than at other times of day .

The potential for crop improvement using circadian-dependent traits

Given that circadian regulation increases the fitness of plants at a number of levels and agronomic traits are associated with certain circadian clock genes, there is significant interest in understanding and optimising circadian networks in crop species. The overall architecture of the oscillator is broadly conserved between the model system *Arabidopsis thaliana* and agronomically important crop species. A number of circadian clock genes underlie QTLs (Quantitative Trait Loci) associated with key agronomic traits, indicating that the circadian-related fitness advantages conferred to Arabidopsis by circadian regulation may also be relevant to crops. Flowering time and biomass accumulation are obvious target traits, but given the widespread influence of the oscillator there may be other aspects of crop productivity that could be improved with increasing understanding of circadian rhythms. One example might be to increase the latitudinal range over which some crops can be grown, by manipulating the role of the circadian clock in photoperiodism. In the face of climate change and associated increases in abiotic stress, the gating of environmental signalling pathways may also become an area of interest to agronomists.

Case study 1: A slower clock was selected for during the domestication of tomato

Analysis of wild and cultivated tomato varieties identified that cultivated varieties have a longer circadian period and delayed phase when compared to wild relatives. QTL analysis isolated two genomic regions that independently controlled the period and phase phenotypes, and the phase QTL was subsequently mapped to a homologue of an Arabidopsis light signalling protein (EID1). Genetic analysis suggests delayed phase was selected for relatively early in tomato domestication, and long period arose at a later stage. The late phase mutation results in late flowering and increased chlorophyll content specifically in long days, both of which would have been advantageous as tomato spread from Mesoamerica to more northerly latitudes.

Case study 2: Photoperiodism in Barley is controlled by a circadian clock component

Barley was domesticated around 8000 years ago in the fertile crescent (between the Mediterranean Sea and Persian Gulf, including modern Iraq, Egypt, Lebanon, Jordan and Israel). Wild barley (*Hordeum vulgare* ssp. *spontaneum* C. Koch.) is a long day plant, so flowering is accelerated in when day lengths are longer than ~13 hours. Cultivated barley (*H. vulgare* ssp. *vulgare* L.) has two forms; winter barley which is sown at the end of the autumn and is harvested in the spring, and spring barley which is sown in early spring and is harvested in the summer.

Growing spring barley has the advantage that sowing occurs after winter, therefore the potential for frost damage is reduced. On the other hand, winter barley has the advantage that harvesting occurs before the height of summer when dehydration may affect yield. There are several differences between the two varieties, one of which is a difference in sensitivity to photoperiod. Winter barleys are photoperiod sensitive, i.e. flowering is accelerated in long days, which is the ancestral phenotype. Spring barleys are photoperiod insensitive, i.e. flowering is not accelerated by long days therefore flowering (and therefore harvest) occurs later in the year. Fine mapping of a cross between the

photoperiod sensitive variety 'Igri' and the late flowering non-sensitive variety 'Triumph' identified a genomic region containing a single pseudo-response regulator gene, which is most similar to the Arabidopsis circadian clock gene *PRR7*. The recessive *ppd-H1* mutation causes a single amino acid change in the protein which results in photoperiod insensitivity, although mutation of this gene does not affect the free-running period of the circadian oscillator. Analysis of barley land races across Europe shows that there is a shift from *Ppd-H1* (photoperiod sensitive) varieties in the south to *ppd-H1* (photoperiod insensitive) varieties in the north, indicating that there has been selection pressure at this locus during the spread of barley away from the fertile crescent.

As flowering time is such an important characteristic of cereal crop yield, the potential to fine tune flowering time to environmental conditions and extend growing seasons is of great interest. PRR7 provides an example of difference between the oscillator model obtained from studies in *Arabidopsis thaliana* and the clock in monocots. AtPRR7 is not a major regulator of photoperiodism in natural Arabidopsis populations, whereas natural mutations at PRR7 in both rice and barley alter the flowering time. Crop development based on the Arabidopsis model of the circadian clock will therefore need to be done with care, to ensure any changes to the circadian clock in crops actually target traits of importance for agriculture.

Summary and Future Directions

Plant circadian biology has seen extraordinary progress in recent years and is the focus of research groups across the globe. It is now clear that the circadian clock influences almost all plant processes in one way or another, which contributes to the significant fitness advantage conferred by the circadian clock. Some of this control is direct, with circadian oscillator components directly controlling output pathways, while other processes are controlled indirectly through circadian-dependent gating of environmental signalling pathways. The complexity of the clock and the diversity of processes that it controls means that we have to consider the circadian clock to be an extensive control network within plant cells, where it is both difficult and arguably meaningless to consider any individual clock component in isolation. The use of mathematical modelling approaches to studying circadian rhythms is now widespread, and helps us analyse the emergent properties of the network such as circadian period and gating.

There are many unresolved questions in circadian biology, some of which require technical innovation to address. Some of the open questions in plant circadian biology that remain include:

- Is the circadian oscillator specialized at a molecular and functional level in each plant cell type and organ, and do these oscillators communicate with each other?
- What are the molecular bases for circadian gating in plants?
- What is the contribution of circadian regulation to the performance of crop plants, and how can we capitalize upon this to increase crop production?
- How does circadian regulation in plants contribute to the dynamics of ecosystems?
- How did the plant circadian oscillator evolve?

The number of papers published on plant circadian rhythms continues to increase every year as more metabolic, physiological and developmental processes that the clock regulates are discovered. The interface between circadian biology and downstream processes is still poorly understood in some cases, particularly those where the gating of environmental signals plays a role. An increased understanding of plant circadian rhythms may result in the optimization of agriculturally important traits, so this aspect of plant biology is of significant future interest to both the scientific community and commercial enterprise. As the circadian clock underpins so many processes in plants it represents both a fascinating and powerful system for understanding coordination in biological systems, and will continue to be actively researched for many years to come.

Recommended Reading:

This is a very large field of research, and is a fast-moving subject. We apologise to those whose work we have been unable to include for reasons of space.

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